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Dose response of sodium nitrite on vasoactivity associated with HBOC-201 in a swine model of controlled hemorrhage

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Abstract: Sodium nitrite ($NaNO_2$) was evaluated in a 55% EBV hemorrhage swine model to mitigate the increased blood pressure due to HBOC-201. Animals were resuscitated by three 10ml/kg infusions of either HBOC-201 or Hextend with and without $NaNO_2$. All vital signs, coagulation and blood chemistry were measured for 2hr. HBOC-201-vasoconstriction was attenuated only after the first 10.8μmol/kg $NaNO_2$ infusion. Complete abolition was obtained with the highest 3 $NaNO_2$ dose, but side effects were observed. There was no reduction in platelet function due to $NaNO_2$. $NaNO_2$ ability to reduce HBOC-201 vasoactivity was transient and 10.8μmol/kg $NaNO_2$ seems an acceptable dose for further investigation.

Keywords: Nitric oxide donors, blood pressure, pre-clinical resuscitation, trauma

INTRODUCTION

Hemoglobin based oxygen carriers (HBOCs) have been proven efficient oxygen delivery solutions when blood is not available. They could be used successfully to improve resuscitation in pre-hospital situations for patients with hemorrhagic shock [1]. Historically, HBOCs evolved from a single tetrameric molecule (DCLHb), that was later proven harmful, to hemoglobin polymers with chemical cross-links such as HBOC-201 (Hemopure, Biopure[1], Cambridge, MA) [2–5]. This laboratory has ample pre-clinical data with HBOC-201 and has conducted multiple trauma studies demonstrating a higher survival rate and a better restoration of vital parameters (i.e. mean arterial pressure (MAP), and oxygen delivery) using HBOC-201 compared to standard resuscitation fluid such as Hextend after hemorrhage [6–9]. We noticed a marked benefit of HBOC-201 in severe poyltrauma models (e.g. liver and brain injury) where survival is low [8].

The adverse event profile of HBOC-201 reported in a Phase 3 trial has prevented further clinical development. One theory suggests that the underlying mechanism for many of the adverse events reported is the vasoactive effect of HBOC-201[10–12]. This vasoactivity, shown to be associated with peripheral vasoconstriction due to nitric oxide (NO) scavenging [13], results in an elevation in arterial blood pressure with potential cardiotoxic events particularly with older patients [12]. The precise mechanism resulting in this vasoconstriction is complex but nitric oxide scavenging has been postulated as the primary cause. [13–17]. The low percent (~3%) of tetrameric and dimeric forms that still subsists in the HBOC-201 polymer solution may cause vasoconstriction [3,13] although lowering the tetrameric content of HBOC-201 did not

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14. ABSTRACT

Sodium nitrite (NaN02) was evaluated in a 55% EBV hemorrhage swine model to mitigate the increased blood pressure due to HBOC-201. Animals were resuscitated by three lOml/kg infusions of either HBOC-201 or Hextend with and without NaNOr All vital signs, coagulation and blood chemistry were measured for 2hr. HBOC-201-vasoconstriction was attenuated only after the first 1 O.SJ.Lmollkg NaN02 infusion. Complete abolition was obtained with the highest 3 NaN02 dose, but side effects were observed. There was no reduction in platelet function due to NaN Or NaN02 ability to reduce HBOC-201 vasoactivity was transient and 1 O.SJ.Lmollkg NaN02 seems an acceptable dose for further investigation.

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affect systemic pressure in swine [9]. Another hypothesis suggests that HBOCs with high P50 deliver excessive O_2 to tissue resulting in vasoconstriction [18].

The deoxygenated heme-globins (hemoglobin, myoglobin, neuroglobin, cytoglobin) are allosterically functional nitrite reductases that mediate physiological and pathological hypoxic NO signaling. NO is a vasorelaxant agent acting as a regulator of vasoactivity and blood flow. It is produced by endothelial cells via NO synthetase (cNOS) and stimulated by calcium release under normal physiologic conditions and normal blood flow rate. The relaxation of the smooth muscle consequently causes vasodilation and prevents platelet aggregation. These conditions change in stress situations when NO is massively produced above basal conditions. NO has a short lifespan in plasma (seconds) and it is readily transformed into nitrite. If hemoglobin is present extracellularly in the plasma layer (e.g. HBOC-201 infusion, hemolysis in sickle cell disease), NO will be depleted at a faster rate than when encapsulated in RBC, thus resulting in vasoconstriction [19–21].

To offset NO scavenging due to HBOC, the addition of NO donors such as sodium nitrite ($NaNO_2$), L-arginine, nitroglycerine, or sodium nitroprusside are currently being examined in this laboratory with sodium nitrite ($NaNO_2$) as the initial candidate. $NaNO_2$ has been shown to reduce clinical symptoms of free plasma hemoglobin in diseases such as sickle cell by preventing vasoconstriction [20, 22–24]. In a mouse model, pretreatment with a bolus of 30 μMol $NaNO_2$ prevented the subsequent systemic hypertension due to tetrameric Hb infusion with only a slight elevation in methemoglobin (HbFeIII) (MetHb) [25].

We tested the hypothesis that $NaNO_2$ would reduce systemic and pulmonary hypertension caused by resuscitation with HBOC-201. The goal of this study was to use a dose response study to establish the optimal dose of $NaNO_2$ required to mitigate the initial vasoconstrictive effect of HBOC-201. The study was performed with $NaNO_2$ concomitantly administered with HBOC-201 as a resuscitation fluid in a prehospital phase following a 55% estimated blood volume (EBV) controlled hemorrhage.

MATERIALS AND METHODS

The experiments were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals" Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. The study was approved by the WRAIR Institutional Animal Care and Use Committee (IACUC) and all procedures were performed in an animal facility approved by the Association for Assessment and Accreditation for Laboratory Animal Care International (AAALAC).

Animal model: As previously described, 55% of the EBV was removed from Yorkshire swine (20-30 kg) prior to fluid resuscitation [7]. EBV was calculated as 65 ml/kg of body weight. Briefly, swine (n = 36) were anesthetized (ketamine/isofluorane induction and isofluorane maintenance), intubated, and allowed to breath spontaneously (FiO$_2$ = 21%). Rectal temperature was monitored and maintained at 37–39°C using a BAIR hugger device (Model 505, Bair Hugger, MN). The femoral and external jugular veins and carotid artery were catheterized by open technique for vascular access and continuous blood pressure monitoring. A pulmonary artery catheter was inserted. After an equilibration period (5-10 minutes), swine were hemorrhaged to 55% EBV by catheter withdrawal of blood over 15 minutes. Shed blood from the animal was collected in blood bags containing standard anticoagulant (CPD-A) (Fenwal, Deerfield, IL) and later used for autologous transfusion, when necessary. At 15 minutes, swine entered the resuscitation phase and received up to three possible resuscitation fluid infusions at T15, T30 and T45 if prospectively defined criteria were met (i.e. MAP < 60 mm Hg or heart rate (HR) > 5% above baseline). Fluid resuscitation consisted of administration of 10 ml/kg HBOC-201 or Hextend (HEX) via the right jugular vein with or without concurrent $NaNO_2$ over 10 minutes followed by 5 minutes for equilibrium period (e.g. each infusion period was 15 min). The $NaNO_2$ was administered separately via one of the Swan Ganz catheter ports using a syringe pump (AL-1000, World precision Instruments, Fl) at a rate of 1.5 ml/min. There was no contact of the two products prior to systemic mixing in the blood of the animals.

Treatment groups and fluid infusion: Dose response for $NaNO_2$ was based on an earlier study in which 0.27 μmol/min/kg of $NaNO_2$ was infused over 10 min in the dog [26]. A pilot study using swine eligible for three HBOC-201/$NaNO_2$ infusions at T15, T30 and T45 with the 0.27 μmol/min/kg dosage infused over 10 min (i.e. 2.7 μmol/kg $NaNO_2$) indicated no effect. We chose to start the escalation by doubling this dose (i.e. 5.4 μmol/kg $NaNO_2$ (1X)) and the treatment groups are summarized in Table 1. Our treatment groups consisted thus of HB-0 (no $NaNO_2$), HB-1X (3 infusions of 5.4 μmol/kg $NaNO_2$; a cumulative dose of 16.2 μmol $NaNO_2$/kg), HB-2X (3 infusions of 10.8 μmol/kg $NaNO_2$; a cumulative dose of 32.4 μmol/kg $NaNO_2$), HB-3X (with incremental infusions of 8.1, 16.2, and 24.3 μmol/kg $NaNO_2$ and a cumulative dose of 48.6 μmol/kg $NaNO_2$), the colloid control HEX-0 (no $NaNO_2$) and HEX-2X (3 infusions of 10.8 μmol/kg $NaNO_2$, a cumulative dose of 32.4 μmol/kg $NaNO_2$). At T60, the swine entered the "hospital phase" and were eligible to receive autologous blood transfusions at T60, T75, T90 and T105 for Hb < 7 g/dL or crystalloid fluid (saline) for hypotension (MAP < 60 mm Hg). At 2 hours, the swine were euthanized.

Table 1. NaNO$_2$ Dosage for infusion; total amount infused at T60. Animal survival rate

55% Controlled Groups	n	Single doseNaNO$_2$ μmol/kg	Cumulative NaNO$_2$ at T60		NaNO$_2$ per HBOC at T60		Survival Rate (%)	Time (min)	p
			μmol/kg	Mg/kg	μmol/ml HBOC	μg/ml HBOC			
HB-0	6	0	0	0	0	0	100	120	
HB-1X	6	5.4	16.2	1.12	0.54	37.3	100	120	
HB-2X	6	10.8	32.4	2.23	0.54	37.3	100	120	
HB-3X	6	16.2^a	48.6	3.35	0.54	37.3	100	120	
HEX-0	5	0	0	0	–	–	80	105	NS
HEX-2X	7	10.8	32.4	2.23	–	–	86	107	

^{a}HB-3X was given as 8.1 μmol/kg first injection, 16.2 μmol/kg second injection, 24.3 μmol/kg third injection.

HEX is 6% hydroxy-ethyl starch (MW = 670 Kd) prepared in balanced Lactated Ringer's solution (LR) (50:50 racemic mixture, 28 mEq lactate), containing glucose (1 g/L), with a pH of ~6.6, an osmolality of 307 mOsmol/kg, and an oncotic pressure of 30 mmHg (Hextend, Abbott Laboratories, Abbott Park, IL). HBOC-201 is prepared in a buffer similar to LR containing a 50:50 racemic D-and L-lactate mixture (27 mEq lactate), N-acetyl-polycysteine (0.17%), ~13 g Hb/dL, with an oncotic pressure of 17 mmHg, an osmolality of ~300 mOsmol/kg, a pH of ~7.8, and an oxygen affinity (P$_{50}$) of ~38–41 mmHg; being higher than human blood. HBOC-201 does not contain glucose. NaNO$_2$ powder (NIH pharmacy, Bethesda, MD) (100 mg, MW = 69) was diluted with sterile water to a final concentration of 1.5 μg/μl (22 mM) and future adjustment to meet the required concentration were made with sterile water before the infusion.

Animal monitoring: Standard hemodynamic parameters (MAP, HR, cardiac output (CO), and mean pulmonary arterial pressure (MPAP)) were monitored continuously for the entire experiment (2 hours). Arterial lactate (Lac), pH, base excess (BE), oxygen saturation (SaO$_2$), hemoglobin (Hb) MetHb and mixed venous oxygen saturation (SvO$_2$) were measured every 15 minutes until 2 hours (ABL 705, Radiometer, Copenhagen, Denmark). Transcutaneous tissue oxygenation (TCOM) was noninvasively measured with a TCM4 Tina monitor (Radiometer, Copenhagen, Denmark) using 4 Clark type polarographic electrodes (data represent mean values) positioned bilaterally on the upper torso and on the inner thighs. Only gross necropsy was performed on animals without collection of tissue for histopathology in this initial study.

In vitro measurements: All functional laboratory assays were performed at 37°C, consistent with recorded animal temperatures. Thrombosis and hemostasis was assessed as previously described on blood samples collected at T0, 15, 60 and 120 min [27]. A CBC was performed using a Pentra 60C+ cell counter (Horib-aABX Diagnostics, Irvine, CA). Coagulation parameters, including prothrombin time (PT) partial thromboplastin (PTT) and thrombin time (TT), were measured using clot based principles and colorimetric determination on

a Stat Compact (Diagnostica Stago, Parsippany, NJ). Thrombin-Antithrombin (TAT) was measured by using ELISA (Enzygnost, Dade Behring). Thromboelastography (TEG) reaction time (TEG-R), kinetics of clot formation (TEG-K and TEG-α corresponding to deposition of platelets on newly formed fibrin strands), maximum amplitude (TEG-MA), and fibrinolysis (TEG-Ly) were measured using a TEG 5000 instrument (Haemoscope Corp, Niles, IL). The test was initiated with 340 μl whole blood recalcified with 20 μl of CaCl$_2$ CaCl$_2$. In vitro bleeding time was measured by the closure time (PFA) of an ADP-collagen coated capillary after aspiration of 800 μl citrated whole blood using a PFA-100 analyzer (Dade Behring, Deerfield, IL). ADP-induced aggregation was performed adding 7 ul of 1 mM ADP) to 400 μl of whole blood stirred at 1200 rpm using a impedance microprobe to detect aggregate formation (ADP: 17 μM final concentration) (Aggregometer, Chronolog, PA). Aggregation was calculated from recorded traces as percent (%) change in impedance from baseline. Indirect measurement of NO generated in the blood, following the addition of sodium nitrite, was obtained by blood nitrite measurement.

Immediately after collection, whole blood (600 μl) was mixed with a ferricyanide based stop solution (150 μl) to oxidize hemoglobin to MetHb, such that it would not rapidly react any further with NaNO$_2$. Stop solution containing detergent to lyse RBC was used for total nitrite measurement and a stop solution without detergent was used for plasma nitrite measurement. The samples were centrifuged at 14,000 RPM for 2 min and the supernatant was stored at −80°C. At the time of the measurement, samples were thawed at room temperature, diluted with ice cold methanol (1:1, v/v). Nitrite, S-nitrosothiols, Fe-nitrosyls and/or N-nitrosamines were reduced to NO gas after processing by tri-iodide-based gas phase chemiluminescence using a Siever's nitric oxide analyzer (NOA) model 280i (Boulder, CO 80301) as described by Braman and Hendrix [28].

Data Analysis and Statistics: Results are presented as means ± standard deviation (SD). Six animals per group provide a power of 88% to detect a 10 mmHg difference

in MAP. Survival rates and time-to-events were analyzed with Fisher exact test and Kaplan Meier test for censored non parametric data, respectively. For multiple variables and for data collected over time, results were analyzed using the mixed statistical model for global inspection of continuous measurements (Proc Mixed, SAS, Cary, NC). Significant group and/or time effects were indicated and, when appropriate, individual measures were subsequently compared using a two-tailed paired Student's t-test assuming equal variance. A p ≤ 0.05 was considered significant.

Results

Survival and physiology: Pig weights (25.7 ± 3.1 kg) were similar for all groups (ANOVA). Survival to 2 h was not different among groups (Fisher's exact). Survival was 100% (6/6) with HBOC-201 and HBOC-201 + NaNO$_2$ resuscitation regardless of the NaNO$_2$ dose and one animal died in each of the HEX groups (1/5 and 1/7 for HEX and HEX + NaNO$_2$, respectively) (Table 1). Death of the animals in the HEX groups was unlikely related to treatment: one animal stopped spontaneously breathing at T15 and, per resuscitation protocol, supportive ventilation was not provided; the other suffered the consequences of early severe hemorrhagic shock, remaining hypotensive and tachycardic despite fluid resuscitation.

Clinical course after simulated hospital arrival at T60 was uneventful and similar to animals previously treated with HBOC-201 alone [7]. Although HBOC plus NaNO$_2$ animals survived the 2 h experiment, animals in the HB-2X and HB-3X groups had congested lungs visible at necropsy and one animal in the HB-3X group showed severe petechiae (Figure 5) and pulmonary hemorrhage, not observed in the HEX-2X group.

Simulated pre-hospital fluid resuscitation: All animals in all groups received the maximum 3 resuscitation infusions. The cumulative NaNO$_2$ dose received, the calculated concentration infused, and the concentration relative to HBOC-201 is presented in Table 1.

Simulated in-hospital blood transfusion and saline infusion (Figure 1): From all the possible blood transfusions in the HBOC groups (4 x 6 animals x 4 groups), only one blood transfusion was necessary (HB-1X) (~1%) whereas all blood transfusions (100%) but one were required in the HEX groups (Figure 1A). None of the HEX-treated animals required saline infusion (0%) whereas most HBOC-201 treated-animals received saline infusions (~85%) (81 infusions on the 96 possible) (Figure 1B). There was a significant difference between HBOC-201 and HEX groups (p < 0.01) but none due to NaNO$_2$ regarding blood transfusion and saline infusion.

Hemodynamics (Figure 2): MAP (Figure 2A) was reduced after hemorrhage to 17 ± 5 mmHg comparable in all groups and increased immediately with the first fluid infusion. Within 3 min after onset of first infusion MAP raised sharply above 30 mmHg for HB-0, HB-1X, and HEX-0-treated animals whereas in HB-2X, HB-3X and HEX-2X groups MAP stayed below this mark. At the end of the first infusion (T30), MAP in HB-0 and HB-1X animals reached 55 mmHg and was significantly higher than the HB-2X and HB-3X groups (54 ± 18 mmHg vs. 33 ± 9 mmHg respectively, p < 0.001) where vasorelaxation due to NaNO$_2$ was apparent. After the second infusion (T45), MAP in the HB-2X group increased towards the MAP of the HB-0 and HB-1X groups and reached a similar pressure after the third infusion (86 ± 10 mmHg at T60). MAP of the HB-3X group remained lower than the other HBOCs groups and comparable to HEX-0 (p < 0.05; time*group). At 2 h, MAPs in HEX-0 and HEX-2X groups were the lowest but slowly trending toward the HBOC group MAPs.

Baseline HR (Figure 2B) was comparable in all groups (120 ± 20 bpm) and increased equally by the end of hemorrhage (T15) to 155 ± 33 bpm indicating tachycardic compensation. HR continued to increase following
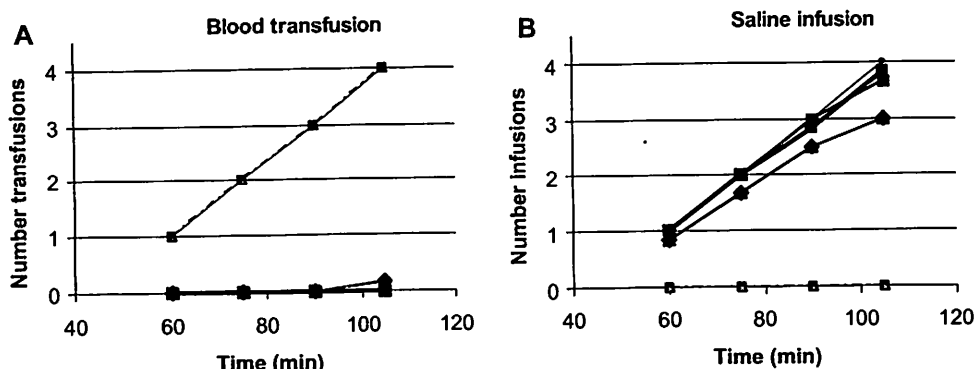

Figure 1. Blood and fluid requirement, A) blood and B) saline, during the simulated hospital phase following resuscitation of animals with — □ — HB-0, — ♦ — HB-1X, — ▲ — HB-2X, — ▨ —HB-3X, - - □ - - HEX-0 and - - △ - - HEX-2X. after a 55% EBV controlled hemorrhage.
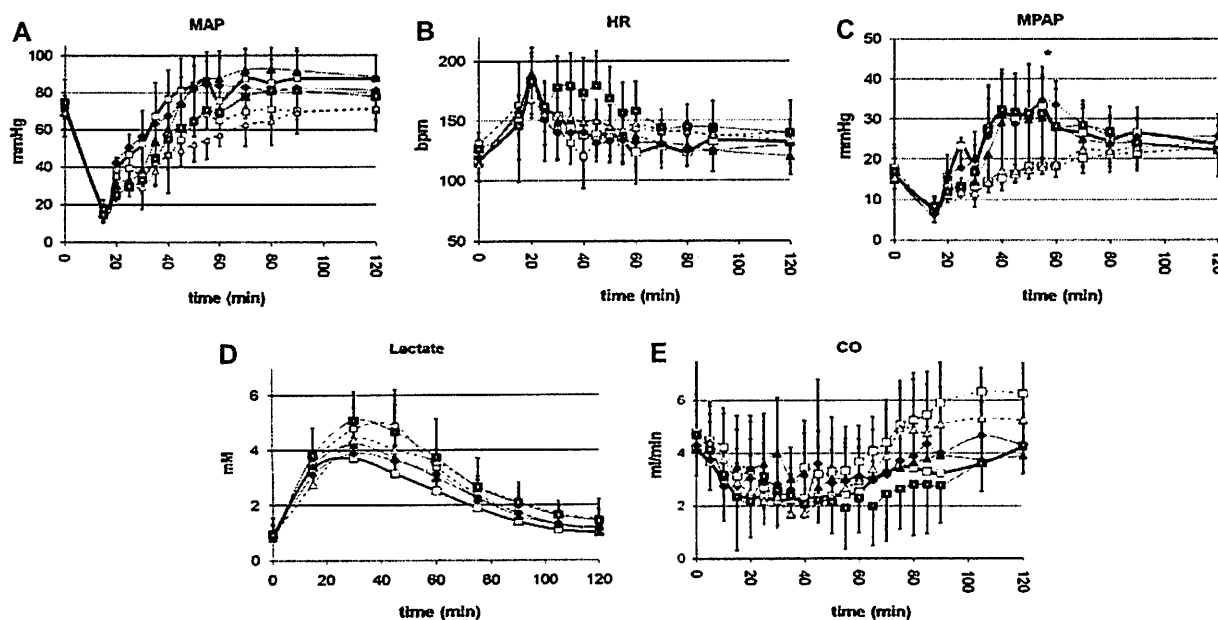
Figure 2. Vital signs illustrated with A) MAP, B) HR, C) MPAP, and D) Lactate, after a 55% EBV controlled hemorrhage in swine (T0) during the 2h experiment: (T0) onset of injury, (T15) resuscitation of animals with: — ☐ — HB-0, — ♦ — HB-1X, — ▲ — HB-2X, — ✖ —HB-3X, - - ☐ - - HEX-0 and - - △ - - HEX-2X and (T60) simulated hospital phase.

the first fluid infusion to a peak – T18 (191 ± 37 bpm) and then HR sharply decreased after the second infusion in all groups except in the HB-3X group that had a marked increase in HR between T30 and T60 (176 ± 30 bpm). HR slowly decreased in all groups to reach 142 ± 23 bpm thereafter. There was no difference between groups at the endpoint.

MPAP (Figure 2C) was reduced below 10 mmHg after hemorrhage. After the first infusion, MPAP increased rapidly in all groups but was higher at T20 for HB-0 and HB-1X (16 ± 6 mmHg) compared to HB-2X and HB-3X (11 ± 3 mmHg; p < 0.01) and comparable to both HEX-0 and HEX-2X. MPAP in all HBOC groups continued to increase equally after T30 to a peak value of 30 ± 9 mmHg, except in the HEX groups where it increased only slightly to 17 ± 3 mmHg at T45. After the third infusion, all HBOC groups remained at a plateau with MPAP around 30 mmHg until – T60 when MPAP began to decrease and there was no difference for any NaNO₂ doses added to HBOC-201. During this time in HEX-treated animals MPAP remained low and similar with or without NaNO₂. By 2 h, all groups had a comparable MPAP of 23 ± 4 mmHg. Overall, only the first infusion of the higher NaNO₂ doses (HB-2X and HB-3X) was able to attenuate elevations in pulmonary pressures.

CO was comparable in all animals at T0, decreased with blood loss and remained low after fluid resuscitation regardless of the treatment. CO started to increase at hospital arrival due to blood and/or saline infusion, thus increasing more rapidly in HEX- than HBOC-treated animal as HEX-animals received blood (colloid) and

HBOC-treated animals received saline (crystalloid). There was no significant difference due to NaNO₂ but there was a trend for a lower CO with the highest NaNO₂ dosage.

Indirect and Direct Measurements of Tissue Oxygenation (Figure 2D): Lactate rose after hemorrhage to peak at T30 and slowly decreased with resuscitation. Lactate after resuscitation with HB-0 tended to be lower than with HEX-0 (3.7 ± 1 vs 4.8 ± 1.5 mM respectively) at T30 without reaching significance. Lactate level increased accordingly with the dose escalation of NaNO₂ when added to HBOC-201. The highest lactate was observed with HB-3X > HB-2X > HB-1X peaking, respectively, at 5.1 mM, 4.2 mM, 3.9 mM at T30. This trend was not statistically different. Results with TCOM (Figure 2F), expressed as a percentage relative to baseline, indicated that tissue oxygenation decreased after hemorrhage and increased after resuscitation. Overall, TCOM trended higher in HBOC-201 groups (HB-0 > HB-1X > HB-2X) than HEX during resuscitation except for the HB-3X that was similar to HEX-0. By the end of the study, TCOM values were 247 ± 153, 187 ± 138, 212 ± 189 vs. 77 ± 20, 75 ± 42, 72 ± 13%, respectively, for HBOC, HB-1X, HB-2X vs. HB-3X, HEX-0 and HEX-2X (p<0.01).

Hematology (Figure 3): Hemoglobin (Hb) (Figure 3A) was restored to baseline level with HBOC (9.0 ± 0.3 g/dl) while hemodilution occurred with HEX (3.9 ± 0.2 g/dl, p < 0.05). Hematocrit (Hct) (Figure 3B) as well as red blood cell (RBC) count decreased due to fluid resuscitation (Hct: 11.2 ± 0.9%) without significant difference among groups. Hct and Hb in HEX-treated animals
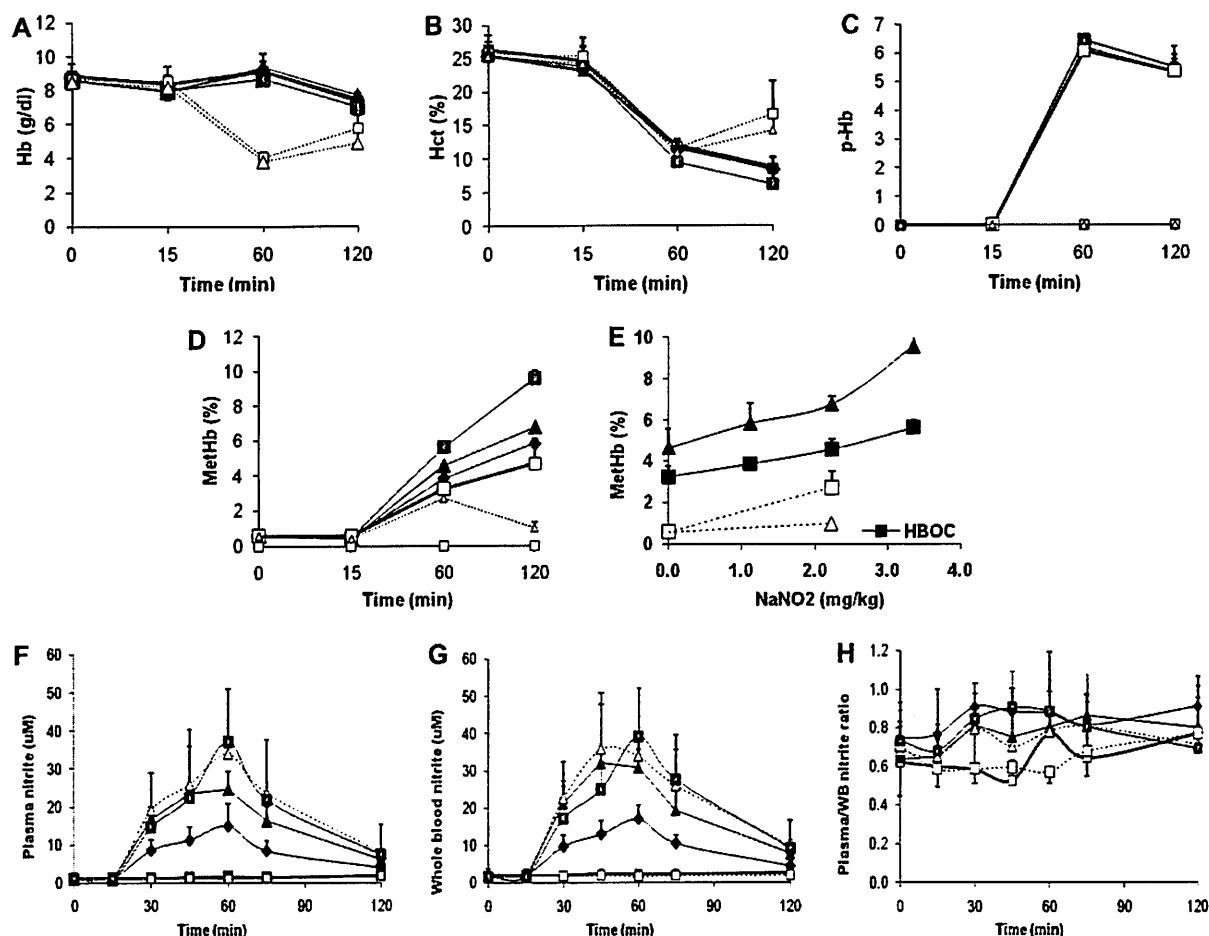
Figure 3. Effect of $NaNO_2$ infusion on the hematology profile in 55% controlled hemorrhage animals resuscitated with: — □ — HB-0, — ◆ — HB-1X, — ▲ — HB-2X, — ⊠ —HB-3X, - - □ - - HEX-0 and - - △ - - HEX-2X. A) Hemoglobin, B), Hematocrit, C) the plasma hemoglobin, D) whole blood MetHb profile, E) Representation of the correlation between MetHb and $NaNO_2$ dose infused at T60 (□) and T120 (△), F) whole blood nitrite and G) plasma nitrite, and H) ratio between plasma and whole blood nitrite.

increased after 60 min when they received blood transfusions (p < 0.05 compared to HBOC groups which did not received blood at this time). The plasma hemoglobin (pHb) (Figure 3C) increased due to the presence of HBOC (not hemolysis) and then declined similarly in all HBOC-treated animals. In this controlled hemorrhage model, no hemolysis was observed in the HEX groups and no hemolysis was assumed in the HBOC groups. There was no effect of $NaNO_2$ on these parameters (Hct, RBC, Hb or pHb). Levels of whole blood MetHb (Figure 3D) increased with increasing numbers of infusions and MetHb continued to increase significantly at T120 in HBOC groups and particularly in HB-3X to 9.8 % (Figure 3E). MetHb increased with $NaNO_2$ concentration; HB-2X and HB-3X were significantly higher than HB-1X group; p < 0.01 (Table 2). In contrast, MetHb in the HEX-2X group increased at T60 and then decreased at T120 after the last infusion. The level of MetHb infused in the HEX-2X group was significantly lower than in the HB-2X group (p < 0.01). This MetHb increase was

significantly (r = 0.99) correlated with the calculated cumulative amount of $NaNO_2$ administered (at T60) and also after the infusion stopped (at T120) (Figure 3 F). Noteworthy, HBOC-201 alone resulted in an initial baseline MetHb of 3.1% and $NaNO_2$ caused additional MetHb production in both HBOC and HEX groups. Level of nitrites was measured in whole blood and plasma (as an indirect measure of NO) and no significant amount of nitrites was found in HEX-0 and HB-0 groups (Figures 3 F and G). After addition of $NaNO_2$, the nitrites peaked at T60 and decreased thereafter. There was a $NaNO_2$ dose dependence on both the whole blood and plasma level of nitrites (15 ± 6 vs. 25 ± 5 vs. 37 ± 5 μM between 1X, 2X and 3X respectively at T60; p < 0.01). Whole blood and plasma nitrites in the 2X dose groups were similar for HEX and HBOC groups. The ratio between plasma and whole blood nitrite was 68 ± 16% before treatment in all samples and remained at this level to the end of the experiment after treatment in the non-nitrite (HEX-0 and HB-0) groups. In contrast, when sodium nitrite was

Table 2. Peak MetHb

55% Controlled	MetHb (%) at T60	p [a]between NaNO₂ doses vs. 0	[b]between HBOC vs. HEX
HB-0	3.2 ± 0.5 [a]		0.01
HB-1X	3.8 ± 0.3 [a]	0.05	
HB-2X	4.6 ± 0.5 [ab]	0.01	0.01
HB-3X	5.6 ± 0.4 [b]	0.001	
HEX-0	0 ± 0		
HEX-2X	2.7 ± 0.8 [b]	0.01	

[a]indicates significance within 0 and other NaNO₂ dose for the HBOC and HEX groups.
[b]indicates significance between HBOC and HEX groups for similar NaNO₂ dose.

infused, the plasma whole blood nitrite ratio was higher and averaged 83 ± 19% (ANOVA, p < 0.01). This suggests a preferential distribution of NO or nitrite in the plasma rather than in the RBC.

Hemostasis (Figure 4): In the pre-hospital phase, platelet count followed the Hct profile and was similar in all groups, indicating no change in platelet number due to NaNO₂ or type of fluid (Figure 4 A). Platelet function as analyzed by PFA (Figure 4 B) did not indicate any significant difference between groups at T60. Increase of PFA was indicative of low platelet count due to dilution. In the hospital phase at T120 PFA showed a non significant decline for the HEX treated animals (likely due to elevation of platelet number due to blood transfusion) and no change for the HBOC's groups except for the

HB-3X group that showed a significant drop that could indicate possible platelet activation or adherence. TEG-K (Figure 4 C), representative of the plug formation with attachment of platelets on fibrin strands, showed no difference between the different HBOC groups regardless of the NaNO₂ addition, indicating no particular interaction of nitrite in this process. In comparison to HBOC groups, HEX group showed a longer kinetics at T60. TEG-MA (Figure 4 D) showed a decrease in the clot strength similar in all groups at T60 related to platelet number. Coagulation measured by PT showed no significant difference among groups and paralleled TEG-R (not shown). PT (Figure 4 E) remained unchanged with HBOC except with HB-3X at T120 where PT decreased at T120 (p < 0.05, compared to T60) as in the HEX groups. Lysis (TEG-Ly) was less than 2% and similar in all groups (not shown). Overall, ADP-aggregation (Figure 4 F) was not significantly different among groups despite the faster decrease in aggregation at T60, with HEX (with or without NaNO₂) compared to HBOC groups but this may be due to the known effect of starch on platelets. Aggregation increased in the HEX groups in hospital phase due to blood transfusion. NaNO₂ did not have any remarkable effect on aggregation as there was no difference between HBOC groups with or without NaNO₂.

Gross pathology: Starting at ~ T45, cutaneous redness was observed at the site of probe applications (e.g., TCOM adhesive patch) on all NaNO₂ + HBOC-201 treated animals indicating either inflammation or a vascular defect. One animal in the HB-3X group had severe diffuse petechiae over its body and grossly hemorrhagic lungs (Figure 5). Surprisingly, this animal survived the
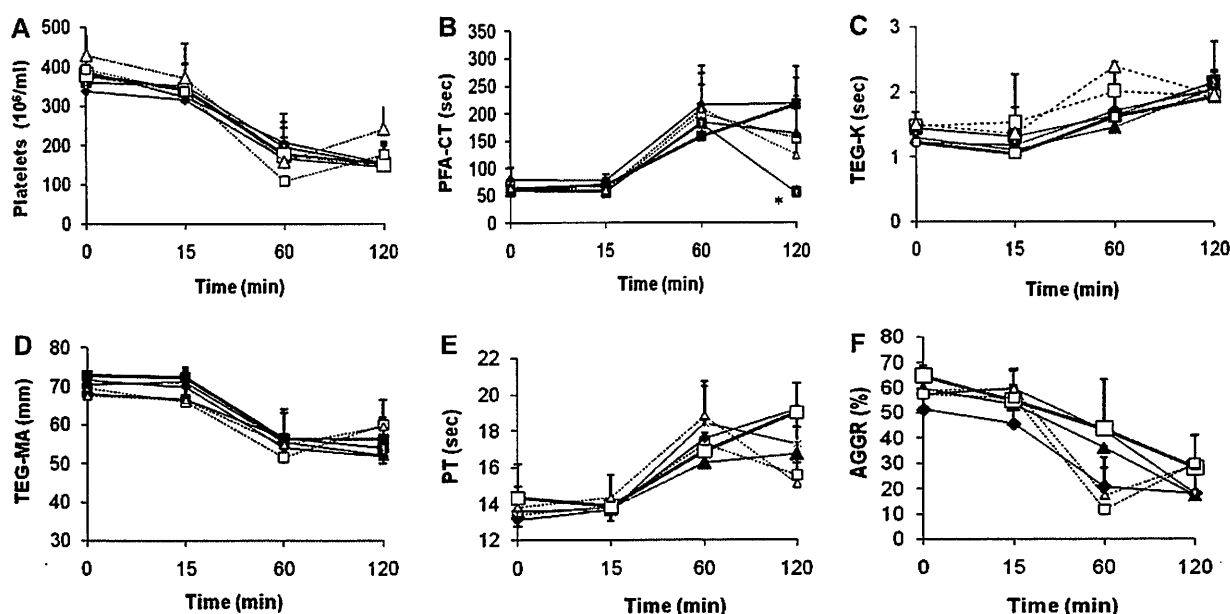

Figure 4. Hemostasis illustrated with A) platelet count, B) Platelet function analyzer (PFA), C) TEG-K, D) TEG-MA,E) PT and F) ADP-aggregation after 55% EBV controlled hemorrhage in swine during the 2h experiment: (T0) onset of injury, (T15) resuscitation of animals with — □ — HB-0, — ◆ — HB-1X, — ▲ — HB-2X, —◼—HB-3X, - - □ - - HEX-0 and - - △ - - HEX-2X.

2 h and none of the vital signs or in vitro data indicated any abnormality; also its platelets and PT were within the normal range and blood gases were comparable to that of other animals in that group. No histopathology was performed on any of the animals.

Discussion

Our laboratory has been evaluating hemoglobin based oxygen carriers for use in hemorrhagic shock in a number of animal models. We generally observe a rapid restoration of blood pressure after infusion of HBOC compared to other colloid resuscitation fluid [7]. One of the possible means to mitigate this overshoot in increase of blood pressure is co-infusion of the NO donor. The present swine model of controlled hemorrhagic shock showed that co-infusion of $NaNO_2$ with HBOC-201 during resuscitation transiently decreased the systemic blood pressure response, and to a much lesser extent pulmonary pressure response, in a dose-related manner. One to three infusions of $NaNO_2$ at doses below 10.8 $\mu mol/kg$ had no significant effects on HBOC-201's systemic or pulmonary hypertension. The minimum effective dose for attenuation of initial systemic hypertension was 10.8 $\mu mol/kg$ but repeat infusions did not have appreciable additive effects. Sustained attenuation was seen only in the group co-infused the highest dose of $NaNO_2$ (HB-3X) where animals received escalating doses from 8.1 to 16.2 to 24.3 $\mu mol/kg$ (average dose 16.2 $\mu mol/kg$ and cumulative dose of 48.6 $\mu mol/kg$ of $NaNO_2$). The same minimum effective dose for attenuation of pulmonary hypertension (10.8 $\mu mol/kg$) but no sustained effects were seen even at the highest doses. Clearly, HBOC-201-induced pulmonary responses were less responsive to $NaNO_2$ than systemic responses.

$NaNO_2$ attenuation of HBOC-201 vasoconstrictive responses was ephemeral. At the end of the first 10.8 $\mu mol/kg$ infusion of $NaNO_2$, 9 μmol of $NaNO_2$ would have been infused per g of HBOC-201 (equivalent to 9 μmol of $NaNO_2$ per 4 μmol Heme from HBOC-201). As HBOC-201 and $NaNO_2$ have different biological half-lives (HBOC-201: ~20 hours [27]; $NaNO_2$: <2 minutes [28, 29]), one can assume that HBOC-201 accumulated faster than NO could be produced in the blood from $NaNO_2$. One can calculate then that the yield in the HB-3X group likely reached 9 μmol $NaNO_2$ per 12 μmol HBOC Heme by the end of the third infusion. Thus, with time, excess plasma HBOC continued to scavenge NO resulting in recurrently elevated blood pressures.

Survival rates were similar with HBOC-201 (100%) and HEX (83%) with or without infusion of sodium nitrite, similar to those in previous studies using the same

animals were allowed to recover for 3 days [7,27]. One animal that in group HB-3X had severe petechiae and hemorrhagic lungs; this animal manifested tachycardia and increased lactic acid and glucose. These serious adverse effects could potentially lead to death in a longer study with endpoint exceeding the present 2 h. There was also a report of nitrite toxicity indicating that sodium nitrite caused side effects ranging from vomiting to death [30]. Cardiac output remained depressed in animals receiving HBOC-201 and the highest doses of $NaNO_2$, presumably due to continued and possibly excessive hypotension. For unclear reasons, in the highest dose group, the animals appeared to compensate with tachycardia after the second infusion, this phenomena was not seen in the HEX groups despite more severe ongoing hypotension. It is possible that high dose $NaNO_2$ had a direct suppressant effect (perhaps through MetHb) on myocardial contractility (but this was not measured). MetHb increased at each addition of sodium nitrite but also continued to T120 at a similar rate as during the infusion. This suggests a persisting presence of nitrite in the blood which continuously induces MetHb production in contact with HBOC only. In our study the maximum methemoglobin averaged around 9.2% well below the critical concerns where methemoglobinia above 30% may cause cyanosis and above 50% may cause seizures and death [20]. Although MetHb did not impair tissue oxygenation, this level could have clinical significance in compromised patients with hemorrhagic conditions and shock. Ironically, the animal that had the petechiae had the lowest metHb in this group (7.8%), did not have symptoms of sickness, and all vital parameters were in normal range. It is possible that oxidative forms other than metHb such as the nitrite accumulation may be responsible for such damaging effects on endothelium.

Whole blood or plasma nitrite pattern are similar and peaked around 60 minutes and then decreased (Figure 3). The ratio of plasma to whole blood was reviewed to analyze the distribution of nitrite between the 2 compartments. Endothelium produced NO is oxidized almost completely to nitrite (NO_2^-) that is stable for several hours in plasma [19], hence the baseline ratio above 0.5. The relatively higher plasma/whole blood nitrite ratio particularly during the second infusion in sodium nitrite-treated groups compared to HEX-0 and HB-0 groups, suggests that nitrite remains distributed in the plasma rather than RBC suggesting a possible increased production of NO to compensate for the vasoconstriction and scavenging. After 60 min where no more HBOC is added, the ratio returned to normal similar to that of the HEX group. A possible explanation is that $HbFe^{2+}$ forms a complex in the presence of NO ($NO + HbFe^{2+} \rightarrow HbFe - NO$) that remains stable longer and may act as a reservoir for

nitrite remaining in plasma. Perhaps sodium nitrite is not acting as an NO donor but also prevents NO to be scavenged [31].

Since nitrite is known to inhibit platelet aggregation by virtue of its conversion to nitric oxide [29], it was anticipated that sodium nitrite would reduce platelet function. We did not observe a decrease in platelet aggregation or inactivation due to NaNO$_2$. Decrease coagulation kinetics and reduced aggregation observed in HEX groups at T60 are possibly due to the hydroxyl starch effect on platelets; Blaicher et al predicted that it would require a 3.8 g/dl HEX solution to reduce aggregation [32]. Within the time frame of this experiment, functional assays did not reveal a significant sodium nitrite effect relevant to platelet or hemostatic effectiveness at the systemic level; reduction of platelet function assays (PFA) was similar at T60. However, in this model there was no vascular injury *per se*, but solely catheter-induced hemorrhage; hence disappearance of platelets by adhesion/aggregation was not expected. The significant reduction of platelets (~50%) and plasma hemostatic factors (~40%-60%) observed resulted only from hemodilution in this model. Thus a effect on platelet function due to NaNO$_2$ could have been concealed by hemodilution. Only the combination of high dose NaNO$_2$ and HBOC (HB-3X) appeared to promote a decreased PFA and PT indicating either a direct chemical effect of NaNO$_2$ or by products on coagulation factors or a response resulting from the physiological/metabolic insult. A number of animals were reported with lung congestion including the animal with petechiae in the HB-3X group. This may be linked to the observation that at T120 with this highest dose of NaNO$_2$ an initiation of coagulopathy may have possibility taken place with also hyper aggregation (low PFA value) but this effect was masked clinically by the low platelet number.

Regarding dosage, in a mouse model Rodriguez gave a bolus of 100 µl nitrite after 90 min of hemorrhagic shock at concentrations equivalent to 0.5, 1.5, 5 and 500 µmol/kg^2 in our model [25]. They reported increased MAP at 2 h with doses of 1.5 to 5 µmol/kg compared to HBOC-201 alone and recommended 1.5 µmol/kg to reduce HBOC-201-induced vasoconstriction. This is in contrast with our model that showed no responses on blood pressures after the first injection of our lower dose (5.4 µmol/kg). Both studies seem comparable indicating that metHb remained around 2-3% with doses below 5 µmol/kg but increased progressively to reach 15% with the highest dose (500 µmol/kg). Furthermore, they reported ~ 1 µM plasma nitrite after 1.5 µmol/kg injection while our results showed much higher plasma nitrite (~ 40 µM) with our highest dose; the reason for such a difference is unclear. Although there are many differences in the two models, it is interesting, nonetheless, that they indicate lethal effects with much higher doses (5 mmol/kg) whereas we observed a deleterious effect at 48.6 µmol/kg. Although doses administration as well as species is different, it indicates that high dose of NaNO$_2$ requires caution.

This study has limitations. 1) Only HBOC-201-induced vasoconstriction is addressed and the effect of NaNO$_2$ on other oxygen carriers may differ as the cause and level of vasoconstriction may vary among different products. 2) Although it seems counterintuitive to use a vasodilator with hemorrhagic conditions, results previously published from this group indicated a more rapid MAP increase with HBOC-201 than standard fluids. If vasoconstriction is a manifestation of the given amount of HBOC-201, reduction of vasoconstriction may simply occur by lowering the dose of HBOC-201. This raises a legitimate question in case of hemorrhagic shock: would HBOC-201- induced vasoactivity benefit to be controlled during resuscitation where blood pressure will naturally try to increase or at hospital arrival when the patient is stabilized? 3) This hemorrhage model may induce ischemia, reducing oxygen to tissues, and therefore action of NaNO$_2$ to regulate HBOC vasoconstriction may be injury model dependent and/or species dependent. 4) NaNO$_2$ was chosen as an initial NO donor candidate to study. Other NO donors (i.e., nitroglycerine or nitroprusside) are also under investigation with the expectation to eliminate the adverse events observed in this study (e.g. petechiae with high NaNO$_2$ dose). 5) In addition there may be a safer way to administer NO donors compared to standard IV injection. Yu et al reported that pretreatment with inhaled 80 ppm NO for 1 hour was efficacious in reducing pulmonary hypertension in awake mice and lambs that received HBOC-201, [13, 17, 30]. 6) Lastly, we acknowledge that although HBOC-201 vasoactivity was reported in older patients in clinical trials [12], we used animals that could be considered juvenile.

Conclusion

Addition of NaNO$_2$ showed a transient effect in the reduction of HBOC-201-induced vasoconstriction (manifested as an initial reduction of systemic and pulmonary blood pressures) but repeat infusions provided no beneficial effects. Moreover, HBOC-201 plus high doses of NaNO$_2$ exhibited MetHb elevation potentially affecting animal health. Surprisingly, there was no obvious coagulation or thrombotic abnormalities with NaNO$_2$ doses of 10.8 µmol/kg. Therefore, caution is recommended in evaluating NaNO$_2$ as a NO donor when administered in combination with HBOC-201.

Acknowledgements

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